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Dependence of the phagocytic uptake of polystyrene microspheres by differentiated HL60 upon the size and surface properties of the microspheres

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Abstract

In order to determine whether the microsphere size affects the phagocytic uptake of microspheres in vitro, polystyrene microspheres with diameters of 0.2, 0.5, 1.0, 6.0 and 10 μm were added to a HL-60 differentiated macrophage-like cell culture system. The extent of the uptake was determined by the amount of superoxide generated from activated macrophages by the usage of a chemiluminescence (CL) assay with luminol. The release amount of superoxide was higher when polystyrene microspheres with diameters between 0.5 and 6 μm (i.e. 0.5, 1, and 6 μm were added to the macrophages than that when those with a diameter 0.2 or 10 μm were added. Especially, polystyrene microspheres with an average diameter of 1 μm were most effectively recognized as foreign materials by the macrophages. The effects of the functional groups on the microsphere surfaces upon the uptake by macrophages were studied using polystyrene microspheres with an average diameter of 1 μm, having primary amine, sulfate, hydroxyl, or carboxyl groups on their surfaces. Polystyrene microspheres having primary amine groups on their surfaces were more effectively engulfed by the macrophages than polystyrene microspheres having other functional groups. Also, polystyrene microspheres coated with human IgG was more easily recognized as foreign materials than intact polystyrene microspheres.

Keywords: HL60; Macrophage; Phagocytotic; Size; Charge

1. Introduction

Much attention has been focused on lattices, liposomes, nanospheres, microspheres and microcapsules as drug carriers in the field of drug delivery systems and artificial cells [1,2]. The rapid

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uptake of intravenously injected particulate drug carriers by cells of the mononuclear phagocyte system (MPS) is the main limitation for drug targeting to other sites in a human body. To overcome the uptake of the particles by MPS, surface modification of the particles by polyethylene glycol has been widely studied [3]. The particle size is also considered to play an important role when the particle is recognized as a foreign material by MPS [4].

In the present study, the effects of the size and surface properties of polystyrene microspheres upon the phagocytic uptake of the microspheres by macrophage-like differentiated HL-60 will be discussed. Also, the experimental condition during the differentiation of HL-60, a human promyelocytic cell, induced by the addition of phorbol myristate acetate to HL-60 will be discussed.

2. Experimental

2.1. Materials

Phorbol 12-myristate-13-acetate (PMA) was purchased from Sigma; RPMI 1640 medium, phenolred free eagle MEM (EMEM) medium and phosphate buffer solution containing no Ca^{2+} nor Mg^{2+} (PBS^-) were from Nissui Pharmaceutical, Japan; gelatin originating from bovine bone was from Wako Pure Chemicals, Japan. Luminol was purchased from Tokyo Kasei Organic Chemicals, Japan. Each kind of polystyrene microsphere suspension (Polybeads™ Microspheres), polystyrene microspheres and polystyrene microspheres having primary amine, sulfate, hydroxyl, or carboxyl groups on their surfaces, was purchased from Polysciences, USA. All other chemicals were of reagent grade.

2.2. Cell culture

Culture medium (RPMI-1640 containing 10% fetal calf serum (FCS)) was purchased from Nissui Pharmaceutical. HL-60, a human promyelocytic leukemia cell line, cells were grown in RPMI-1640 medium supplemented with 10% FCS, 100 U/ml penicillin at 37 °C in an atmosphere of 5% CO_2 and 100% humidity.

2.3. Superoxide anion assays

The amount of superoxide, produced from HL-60 cells by the stimulation of PMA and by the addition of polystyrene microspheres, was measured by a chemiluminescence (CL) assay using a Luminometer (UPD-400, Meidensha, Japan). Luminol was dissolved in methyl sulfoxide to be a final concentration of 10 mg/ml (0.056 M). HL-60 cells were cultured in a Luminometer container, the surface of which was modified with gelatin, as follows. Gelatin was dissolved in PBS^- to be a final concentration of 100 μg/ml and the culture containers were washed with the gelatin solution several times at 37 °C. One ml of HL-60 cells containing 5×10^5 cells were cultured in one culture container in phenol red-free medium containing PMA.

Thirty minuets before PMA or polystyrene microspheres being added to the HL-60 cell culture system, 20 μl of the luminol solution was added to 1 ml of HL-60 cell culture system to be the final concentration of 1×10^{-3} M.

2.4. Differentiation of HL-60 to activated macrophage

About 5×10^5 HL-60 cells were incubated in 1 ml of phenolred free EMEM medium in a container for a CL assay, as mentioned above. PMA was dissolved in *methyl sulfoxide* to be 4.0 mM. PMA solution was then added to the cell container to be the final concentration to be 10, 20, 40, and 80 nM. Differentiation was continued for 6, 12, 24, or 48 h at 37 °C in an atmosphere of 5% CO_2 and 100% humidity.

2.5. Opsonization of polystyrene microspheres with human IgG

Human IgG was isolated from human serum using a Protein G-Sepharose (Amersham-Pharmacia Biotech) column. To coat with human IgG, polystyrene microspheres suspendd in PBS^- were incubated at 37 °C with human IgG at a final concentration of 200 μg/ml with gentle shaking. After 1 h incubation, polystyrene microspheres, thus, opsonized with human IgG were washed

three times with PBS^-. In a control experiment, polystyrene microspheres were coated with bovine serum albumin, instead of human IgG.

3. Results and discussion

3.1. Effects of PMA on cell growth and differentiation

Before discussing the phagocytic uptake of microspheres by macrophage-like HL-60 cells, the effects of PMA concentration and the length of differentiation period upon the phagocytic activity of the differentiated cells were studied. When 20 nM PMA was added to the medium, the cells gradually became adherent to the plastic surface of the culture container, as shown in Fig. 1. On the other hand, the control cells grew by 120% during 24 h, as shown in Table 1. Moreover, after 24 h exposure to 20 and 40 nM of PMA, 46 and 34% of cells were adherent to the culture container. Microscopic observation showed that 24 h after the addition of PMA to the culture medium, the majority of HL-60 cells change their morphology, while HL-60 cells were suspended in the medium before the addition of PMA (Fig. 1).

The uptake of polystyrene microspheres into HL-60 cells in the various differentiation stages was studied using a CL assay [5–7]. PMA was added to HL-60 culture medium to be various concentrations (10, 20, 40, and 80 nM) and the differentiation was performed for 6, 12, 24 and 48 h. After the differentiation of HL-60 cells with PMA for a certain period between 6 and 48 h, polystyrene microspheres with a diameter of 1 μm were added to the HL-60 culture medium. Luminol solution was added to the culture system 30 min prior to the addition of polystyrene microspheres. CL counts during 30 s was measured 2 h after the addition of polystyrene microspheres to HL-60 cells. As clearly observed in Fig. 2, CL counts show two peaks in the HL-60 cells after 6 and 24 h of exposure to PMA. By the comparison of the results shown in Fig. 2 with the above-mentioned microscopic observation (Fig. 1), high CL counts obtained after 24 h of exposure to PMA of HL-60 cells are considered to be caused by the uptake of polystyrene microspheres. We have confirmed that the superoxide release from HL-60 after 6 h exposure of PMA, observed in Fig. 2, is caused not by uptake of the polystyrene microspheres into HL-60 cells but by the interaction of PMA with HL-60 cells, as described below.

Fig. 3 shows the amounts of superoxide released from HL-60 cells after PMA exposure for a certain time period between 6 and 48 h without the addition of polystyrene microspheres. When PMA, the concentration of which was higher than

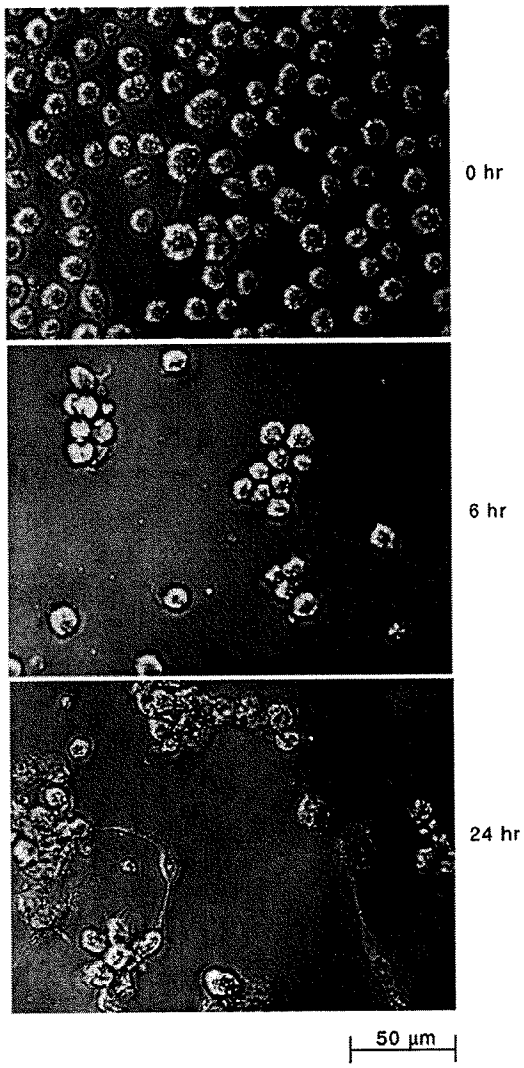

Fig. 1. Morphological changes in HL-60 cells caused by the addition of 20 nM PMA.

Table 1
Numbers of living cells at 0 and after 24 h of PMA exposure

PMA concentration (nM)	Cell count at 0 h	Cell count 24 h later
0	5×10^5	$6.0\times10^5\pm6\times10^3$
20	5×10^5	$2.3\times10^5\pm7\times10^3$
40	5×10^5	$1.7\times10^5\pm1.2\times10^4$

10 nM, was added to the culture medium, the highest CL count was observed 6 h after the addition of PMA and the counts were independent of PMA concentration, while CL was very low when no PMA was added to HL-60 cells. It has been reported that HL-60 cells can release superoxide immediately after stimulation with PMA [5].

In the initial stage of HL-60 cells after exposure of PMA, superoxide may be released from HL-60 not only by the stimulation with PMA, but also by the adhesion of polystyrene microspheres onto the HL-60 cell surfaces. That is, by the comparison of Figs. 2 and 3, effects of polystyrene microspheres upon superoxide production are clearly observed in the initial stage of the differentiation of HL-60 until 6 h exposure of PMA. About 4400 counts of CL were detected for 30 s when polystyrene microspheres were added to differentiated HL-60 cells by the exposure to 10 nM of PMA, as shown in Fig. 2. On the other hand, only about 2800 counts of CL were detected for 30 s when no polystyrene microspheres were added to the system, as shown in Fig. 3. The observed difference by about 1600 CL counts should be caused by the adhesion of polystyrene microspheres onto HL-60 cell surfaces.

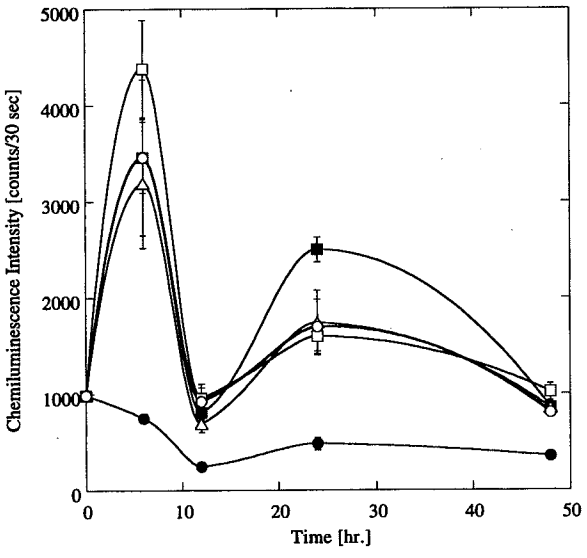

Fig. 2. Superoxide release from HL-60 cells by the addition of polystyrene microspheres. HL-60 cells were differentiated by incubation with PMA. ●, Control; □, 10 nM PMA; ■, 20 nM PMA; △, 40 nM PMA; ○, 80 nM PMA was added at time = 0. The data are from three experiments, each carried out in triplicate. Bars indicate S.E.M.

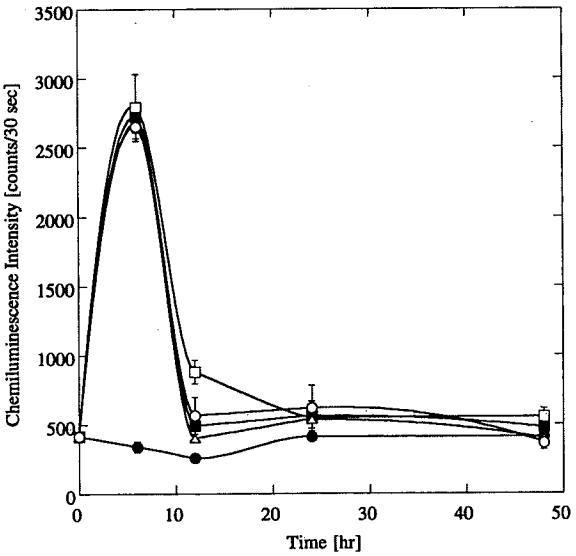

Fig. 3. Superoxide release from HL-60 cells. HL-60 cells were differentiated by incubation with PMA. ●, Control; □, 10 nM PMA; ■, 20 nM PMA; △, 40 nM PMA; ○, 80 nM PMA was added at time = 0. The data are from three experiments, each carried out in triplicate. Bars indicate S.E.M.

We have serially measured CL counts after the addition of PMA to HL-60 cells. As shown in Fig. 4, the CL counts were low in the mixture of luminol and intact HL-60 cells, and in the mixture of luminol, intact HL-60 cells and polystyrene microspheres. Also it was low in the mixture of luminol, 10 nM PMA and HL-60 cells, showing the similar values of CL counts to those observed in Fig. 3. Only when polystyrene microspheres are added to the mixture of luminol, 10 nM PMA and

HL-60 cells, CL counts gradually increase up to about 16 000 counts during 240 min after both PMA and polystyrene microspheres are added to the mixture of HL-60 and luminol. After 240 min, CL counts gradually decrease and then reach to 4400 counts until 360 min (Fig. 2). From these observations, we have decided to use differentiated-HL-60 cells produced by 24 h exposure of 20 nM PMA in the following experiments.

3.2. Effects of particle sizes of polystyrene microspheres

Polystyrene microspheres with diameters of 0.2, 0.5, 1.0, 6.0, and 10.0 μm were added to macrophages differentiated from HL-60 cells in order to observe the dependency of the microsphere uptake by macrophages upon the size of foreign materials. Superoxide was assayed by a CL assay serially after the addition of the microspheres. As clearly observed in Fig. 5, when polystyrene microspheres with diameters of 10 μm were added to the system, the CL intensity was almost the same as that of control (PBS^- solution), suggesting that the microspheres were not engulfed by macrophages. Also, when polystyrene microspheres with diameters of 0.2 μm were added to the system, the CL intensity was low. On the other hand, when polystyrene microspheres having diameters between 0.5 and 6 μm were added, the CL intensity gradually increases. Especially the microspheres having diameters of 1 μm seem to be more effectively trapped by macrophages than the microspheres with other diameters, while, the differences were relatively small.

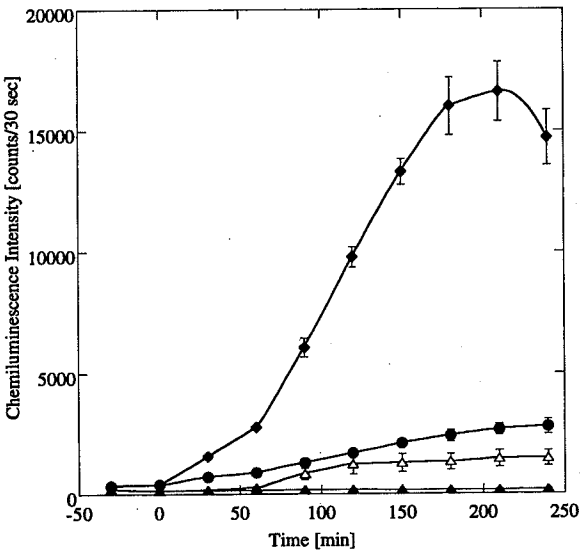

Fig. 4. Superoxide release from HL-60 cells in the initial 4 h ▲, luminol + HL-60 cells; △, luminol + HL-60 cells + polystyrene microspheres; ●, luminol + 10 nM PMA + HL-60 cells; ◆, luminol + 10 nM PMA + HL-60 cells + polystyrene microspheres. The data are from three experiments, each carried out in triplicate. Bars indicate the S.E.M.

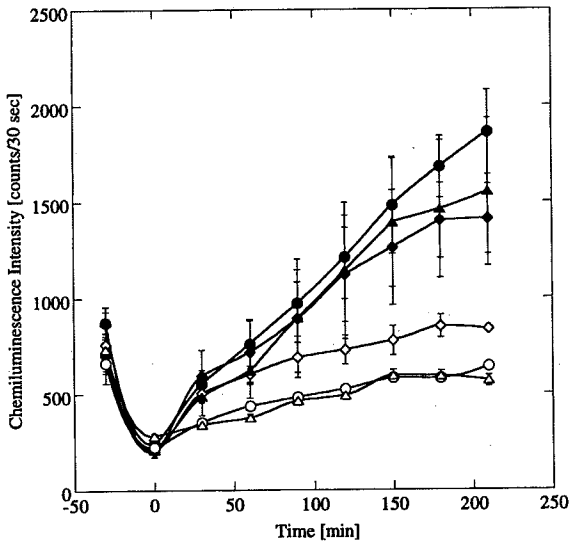

Fig. 5. Effects of sizes of polystyrene microspheres. Polystyrene microspheres with diameters of ◇, 0.2; ◆, 0.5; ●, 1.0; ▲, 6.0; ○, 10.0 μm and △, PBS^- (control) were added to macrophages. The data are from three experiments, each carried out in triplicate. Bars indicate the S.E.M.

3.3. Effects of surface properties of microspheres

In order to observe the effects of surface properties of microspheres upon the efficacy of the uptake of macrophages, polystyrene microspheres with diameters of 1.0 μm, having primary amine, sulfate, hydroxyl, or carboxyl groups on their surfaces were added to macrophages, and then generation of superoxide was serially monitored. Results are shown in Fig. 6. As clearly observed,

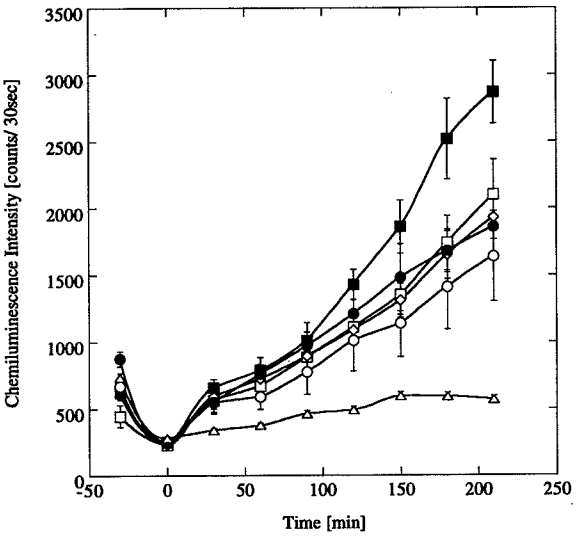

Fig. 6. Effects of functional groups existing on the polystyrene microsphere surfaces. Polystyrene microspheres having ■, primary amine; □, sulfate; ○, hydroxyl; ◇, carboxyl groups on their surfaces; ●, intact polystyrene microspheres, and △, PBS^- (control) were added to macrophages. The data are from three experiments, each carried out in triplicate. Bars indicate the S.E.M.

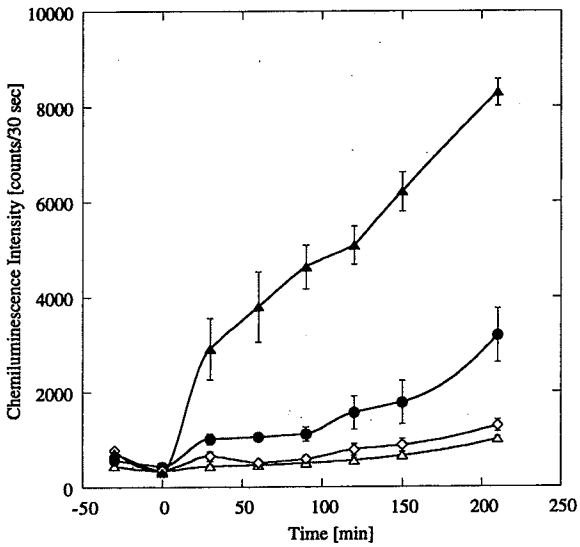

Fig. 7. Effects of opsonization of polystyrene microspheres with human IgG. Polystyrene microspheres opsonized by human IgG (▲), polystyrene microspheres (●), IgG solution (◇), and PBS^- (△) were added. The data are from three experiments, each carried out in triplicate. Bars indicate the S.E.M.

only microspheres having primary amine groups were effectively trapped by macrophages. The average values of the surface charges were negative at pH 7.4 in all kinds of the microspheres, which was confirmed by the measurement of the electrophoretic mobility of microspheres (data are not shown). Therefore, macrophages are considered to detect not the surface charge density of the microspheres but primary amine groups existing on the microsphere surfaces.

3.4. Effects of human IgG

By the opsonization of the polystyrene microsphere surface with human IgG, the microspheres became effectively engulfed by macrophages, as shown in Fig. 7. That is, when polystyrene microspheres, having a diameter of 1.0 μm, the surfaces of which were coated with human IgG, the polystyrene microspheres were more effectively detected by macrophages than the intact polystyrene microspheres.

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